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(12)(19)(CA) Demande-Application

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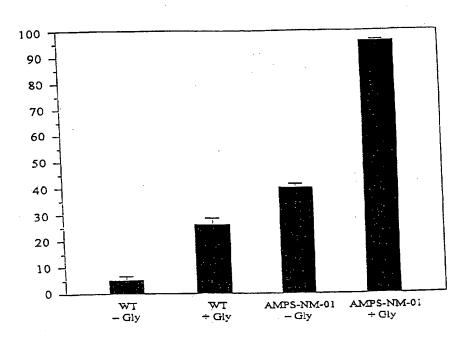
(21) (A1) 2,255,284

(22) 1998/12/17

(43) 1999/06/22

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- (51) Int.Cl. 6 C12N 15/54, C12N 15/80, C12N 1/15, C12N 1/14, C12P 25/00
- (30) 1997/12/22 (197 57 180.8) DE
- (30) 1998/09/09 (198 40 709.2) DE
- (54) ORGANISMES UNICELLULAIRES OU PLURICELLULAIRES UTILISES DANS LA PREPARATION DE RIBOFLAVINE
- (54) UNICELLULAR OR MULTICELLULAR ORGANISMS FOR PREPARING RIBOFLAVIN

Drawings



(57) The present invention relates to a unicellular or multicellular organism, in particular a micro-organism, for biotechnologically preparing riboflavin. This organism is distinguished by the fact that it exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/l.

Abstract of the disclosure

The present invention relates to a unicellular or multicellular organism, in particular a microorganism, for biotechnologically preparing riboflavin. This organism is distinguished by the fact that it exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/1.

Unicellular or multicellular organisms for preparing riboflavin

The present invention relates to a unicellular or multicellular organism for preparing riboflavin using microorganisms.

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Vitamin B2, also termed riboflavin, is essential for humans and animals. Inflammations of the oral and pharyngeal mucous membranes, cracks in the corners of the mouth and pruritus and inflammations in the skin folds, among other damage to the skin, conjunctival inflammations, diminished visual acuity and clouding of cornea appear in association with vitamin deficiency. Cessation of growth and decrease in weight can occur in infants and children. Vitamin B, therefore is of importance economically, in particular as a in association with vitamin vitamin preparation deficiency and as a feed additive. In addition to this, it is also employed as a foodstuff colorant, example in mayonnaise, ice cream, blancmange, etc.

Riboflavin is prepared either chemically or microbially. In the chemical methods of preparation, the riboflavin is as a rule isolated in multi-step processes as a pure end product, with, however, relatively expensive starting compounds, such as Dribose, having to be employed. For this reason, the chemical synthesis of riboflavin is only suitable for those applications for which pure riboflavin is required, for example in human medicine.

Using microorganisms to prepare riboflavin offers an alternative to preparing this substance chemically. Preparing riboflavin microbially is particularly suitable in those instances in which this substance is not required to be of high purity. This is the case, for example, when the riboflavin is to be employed as an additive to feed products. In such

cases, the microbial preparation of riboflavin has the advantage that the riboflavin can be obtained in a one-step process. In addition, renewable raw materials, such as vegetable oils, can be employed as starting compounds for the microbial synthesis.

It is known to prepare riboflavin by fermenting fungi such as Ashbya gossypii or Eremothecium ashbyi (The Merck Index, Windholz et al., eds. Merck & Co.), page 1183, 1983, A. Bacher, F. Lingens, Augen. Chem. 1969, p. 393); however, yeasts, such as Candida or Saccharomyces, and bacteria, such as Clostridium, are also suitable for producing riboflavin.

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Methods using the yeast Candida famata are also described, for example in US 05231007.

Bacterial strains which overproduce riboflavin 15 are described, for example, in EP 405370, GB 1434299, DE 3420310 and EP 0821063, where the strains were obtained by transforming the riboflavin biosynthesis genes from Bacillus subtilis. However, prokaryotic genes were unsuitable for a method of 20 riboflavin recombinantly which preparing eukaryotes such as Saccharomyces cerevisiae or Ashbya gossypii. For this reason, the specific genes riboflavin biosynthesis were, as described in 25 WO 93/03183, isolated from a eukaryote, namely from Saccharomyces cerevisiae, in order thereby to provide a recombinant method for preparing riboflavin eukaryotic production organism. However, recombinant preparation methods of this nature are either 30 unsuccessful, only enjoy limited orsuccess, producing riboflavin if there is inadequate provision of substrate for the enzymes which are specifically involved in the riboflavin biosynthesis.

In 1967, Hanson (Hanson AM, 1967, in Microbial 35 Technology, Peppler, HJ, pp. 222-250, New York) found that adding the amino acid glycine increases the formation of riboflavin in Ashbya gossypii. However, such a method is disadvantageous because glycine is a

very expensive raw material. For this reason, efforts were made to optimize riboflavin production by preparing mutants.

German Patent Specification 19525281 discloses a method for preparing riboflavin which involves culturing microorganisms which are resistant to substances which have an inhibitory effect on isocitrate lyase.

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German Laid-Open Specification 19545468.5-41 discloses another method for preparing riboflavin microbially in which the isocitrate lyase activity or the expression of the isocitrate lyase gene of a riboflavin-producing microorganism is increased. However, even in comparison with these methods, there is still a need for a further optimization of the riboflavin preparation.

object of the present invention The consequently that of making available a unicellular or multicellular organism, preferably a microorganism, for the biotechnological preparation of riboflavin, which microorganism enables formation of the riboflavin to be further optimized. In particular, an organism should be which suitable available is for preparing made riboflavin while economizing on raw materials and which consequently makes possible a production which is more economical than that of the previous state of the art. In particular, the organism should permit an increased formation of riboflavin, without any addition glycine, as compared with the previous organisms.

This object is achieved by means of a unicellular or multicellular organism which exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the Ashbya gossypii species ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/1.

Culturing under standard conditions means culturing, at 30°C and 120 rpm, in 500 ml shaker flasks possessing two baffles. 50 ml of a solution of 10 g of yeast extract/l containing either 10 g of glucose/l or 10 g of soybean oil/l are employed per flask as the medium. The media are inoculated with 1% of a 16 h culture carried out under the same conditions.

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The objective of this sought-after alteration of the intracellular metabolism of glycine can be achieved using the known methods for improving organism strains. This means that, in the simplest case, appropriate strains can be prepared by means of screening after the selection which is customary in microbiology. It is also possible to use mutation in conjunction with subsequent selection. In this case, the mutation can be carried out either by means of chemical mutagenesis or by means of physical mutagenesis. A further method is that of selection and mutation together with subsequent recombination. Finally, the organisms according to the invention can be prepared by means manipulation.

According to the invention, the organism is altered such that it produces glycine intracellularly in a quantity which is greater than its requirement for maintaining its metabolism. According to the invention, this increase in intracellular glycine production can achieved by preparing an organism in which the activity of the enzyme threonine aldolase is increased. This can be achieved, for example, by increasing substrate turnover by means of altering the catalytic center or by abolishing the effect of enzyme inhibitors. An increase in the activity of the threonine aldolase enzyme can also be elicited by increasing the synthesis of the example by means of enzyme, for amplification or by eliminating factors which repress the biosynthesis of the enzyme.

According to the invention, the endogenous threonine aldolase activity can preferably be increased

by mutating the endogenous threonine aldolase gene. Such mutations can either be produced randomly by means of classical methods, such as using UV irradiation or mutation-provoking chemicals, or in a targeted manner using genetic engineering methods such as deletion, insertion and/or nucleotide exchange.

Increased expression of the threonine aldolase gene can be achieved by incorporating copies of the threonine aldolase gene and/or by enhancing regulatory factors which exert a positive effect on threonine aldolase gene expression. For example, regulatory elements can preferentially be enhanced at the transcriptional level by, in particular, increasing the transcription signals. In addition to this, however, it is also possible to enhance translation by, for example, improving the stability of the mRNA.

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In order to increase the gene copy number, the for example, threonine aldolase gene can, incorporated into a gene construct or a vector which preferably contains regulatory gene sequences which are assigned to the threonine aldolase gene, in particular which enhance gene expression. those sequences riboflavin-producing microorganism is then transformed construct containing the threonine with the gene aldolase gene.

According to the invention, the threonine aldolase can also be overexpressed by exchanging the promoter. In this context, it is also possible to achieve the higher enzymic activity in an alternative manner by incorporating gene copies or by exchanging the promoter. However, it is equally also possible to achieve the desired alteration in the enzymic activity by simultaneously exchanging the promoter and incorporating gene copies.

Since threonine is limiting in an organism which has been altered in this way, it is necessary to feed in threonine when the cell according to the invention is employed. The improved uptake of the

threonine and its virtually quantitative conversion into glycine lead to a surprisingly large increase in riboflavin formation such as was not previously achievable by feeding in glycine. Alternatively, the endogenous formation of threonine in the organism can be increased, for example, by eliminating the feedback resistance of the aspartate kinase.

The threonine aldolase gene is preferably isolated from microorganisms, particularly preferably from fungi. Fungi of the genus Ashbya are once again preferred in this context. The species Ashbya gossypii is highly preferred.

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However, all other organisms whose contain the sequence for forming threonine aldolase, that is animal and plant cells as well, are also suitable for isolating the gene. The gene can be isolated by means of homologous or heterologous compleis defective mentation of a mutant which in threonine aldolase gene or by means of heterologous PCR using heterologous primers. probing or subcloning, the size of the insert in the complementing plasmid can subsequently be reduced to a minimum by means of suitable restriction enzyme steps. After the putative gene has been sequenced and identified, subcloning which gives an accurate fit is effected by means of fusion PCR. Plasmids which carry the resulting fragments as inserts are introduced into the threonine aldolase gene-defective mutant, which is then tested for the functionality of the threonine aldolase gene. Functional constructs are finally used to transform a riboflavin producer.

isolation Following and sequencing, the threonine aldolase genes can be obtained with nucleosequences which encode the given amino acid sequence or its allelic variation. Allelic variations particular, derivatives which include, in obtained inserting or substituting by deleting, nucleotides from appropriate sequences while at the

same time retaining the threonine aldolase activity. A corresponding sequence, from nucleotide 1 to nucleotide 1149, is shown in Figure 2b.

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A promoter having the nucleotide sequence from nucleotide -1231 to nucleotide -1 as depicted in the abovementioned sequence, or a DNA sequence which has essentially the same effect, is, in particular, placed upstream of the threonine aldolase genes. Thus, a promoter which differs by one or more nucleotide substitutions, by insertion and/or by deletion from the promoter which possesses the nucleotide sequence shown without, however, the functionality or the activity of the promoter being impaired, can, for example, be placed upstream of the gene. In addition, the activity of the promoter can be increased by altering its sequence, or the promoter can be completely replaced by active promoters.

regulatory Moreover, gene sequences or regulatory genes which, in particular, increase the activity of the threonine aldolase gene can be assigned to the threonine aldolase gene. Thus, enhancers, which increase threonine aldolase gene expression improving the interaction between the RNA polymerase and the DNA, can, for example, be assigned to the threonine aldolase gene.

One or more DNA sequences can be placed upstream and/or downstream of the threonine aldolase gene, which does or does not possess an upstream promoter or does or does not possess a regulatory gene, such that the threonine aldolase gene is contained in a gene structure. Plasmids or vectors which contain the threonine aldolase gene and are suitable for transforming a riboflavin producer can be obtained by cloning the threonine aldolase gene. The cells which can be obtained by transformation contain the gene in replicatable form, i.e. in additional copies in the chromosome, with the gene copies being integrated at

arbitrary sites in the genome by means of homologous recombination.

The objective, according to the invention, of partial or complete intracellular formation of glycine can also be achieved by preparing organisms in which the intracellular degradation of glycine is at least partially blocked. Mutations of this nature can, as already described above, either be generated in a random manner by means of classical methods using physical or chemical mutagenesis, for example using UV irradiation or mutation-provoking chemicals, or in a targeted manner by means of genetic engineering methods.

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According to the invention, the objective of the increased intracellular formation of glycine can preferably be achieved by altering the gene for serine hydroxymethyltransferase. Such alterations can, for example, be achieved by mutations, such as insertions, deletions or substitutions, in the structural gene or the regulatory elements, such as promoters and transcription factors, which are associated with this gene.

According to the invention, it was observed, surprisingly, that these mutants include mutants which are resistant to glycine antimetabolites. The glycine antimetabolite-resistant mutants which are preferred are those unicellular or multicellular organisms which are resistant to alpha-aminomethylphosphonic acid and/or alpha-aminosulfonic acid.

This can, for example, be achieved in exactly the same way by selecting mutants which are replaced by the threonine structural analog β -hydroxynorvaline and/or which are substituted at the threonine and/or lysine analogs.

Consequently, mutants which can be employed in accordance with the invention can also be prepared by appropriate selection. Such resistant unicellular or multicellular organisms can therefore be prepared using the classical screening methods which are in general use in microbiology.

In the organisms described, riboflavin production can be further increased if the export into the medium of the glycine which is formed intracellularly is at least partially blocked. In the simplest case, it is sufficient to supplement with glycine in order to achieve this. As an alternative, the carrier which is responsible for the export can be switched off by disrupting the gene.

In addition, an increase in intracellular glycine concentration can be achieved by altering the glyoxylate metabolism, e.g. by increasing the activity of glyoxylate aminotransferase. Another option is to optimize the synthesis of intracellular glycine from carbon dioxide and ammonia.

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In summary, it can be stated that the object according to the invention can preferably be solved by increasing intracellular synthesis of the glycine, at least partially blocking degradation of the glycine, at least partially inhibiting transport of the glycine out of the cell, altering the glyoxylate metabolism and optimizing glycine synthesis from ammonia and carbon dioxide. These solutions can be used as alternatives, or cumulatively or in any arbitrary combination.

An additional increase in riboflavin formation can be achieved by adding glycine to the nutrient medium.

The unicellular or multicellular organisms which are obtained in accordance with the invention may be any arbitrary cells which can be employed for biotechnological processes. Examples of these cells are fungi, yeasts, bacteria and plant and animal cells. In accordance with the invention, the cells are preferably transformed fungal cells, particularly preferably fungal cells of the genus Ashbya. The species Ashbya gossypii is particularly preferred in this context.

In that which follows, the invention is explained in more detail with the aid of examples,

without this being associated with any restriction of the invention to the subject matter of the examples:

Example 1

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- Selecting a mutant which is resistant to alpha-amino-methylphosphonic acid (AMPS).

Ashbya gossypii spores were mutagenized with UV light. The spores were then added to plates treated with 70 mM alpha-aminomethylphosphonic acid. Inhibition of riboflavin formation can be recognized by the fungus forming yellow colonies without inhibition and white colonies with inhibition. Accordingly, the yellow organisms, i.e. those which were resistant to the inhibitor, were isolated. This method was used to obtain the resistant strain AMPS-NM-01, inter alia.

Experiments carried out on plates containing 200 mM AMPS demonstrated that this strain still exhibited a yellow colony color, in contrast to the starting strain, which remained completely white. In submerged culture, the mutant exhibited the same formation of riboflavin in the absence of glycine as did the wild type in the presence of glycine (cf. Figure 1).

Investigations of the specific enzymic activities of 25 mutant showed that wild type and hydroxymethyltransferase activity was reduced by 50% (Fig. 7). Since it was possible to demonstrate by feeding 13C-labeled threonine that formation of serine, catalyzed by serine presumably 30 which is place from glycine hydroxymethyltransferase, takes (Table 1), the increase in riboflavin formation can be explained by a reduction in the quantity of glycine draining off to form serine.

The composition of the minimal medium used in Table 1 is as follows:

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	Solution A: (100 times)	KH₂PO₄	200	g/l	pH 6.7 with KOH
	Solution B:	NH4Cl	15	g/l	
5	(10 times)	Asparagine	5	g/l	
		NaCl	2	g/l	
		$MgSO_4 \times 7H_2O$	4	g/l	
		$MnSO_4 \times H_2O$	0.5	g/l	
		ClCl ₂ × 2H ₂ O	0.4	g/l	
10		Myoinositol	1.0	g/l	
		Nicotinamide	2.5	g/l	
		Yeast extract	2	g/l	
	C source:	Glucose or			
		soybean oil	2.5	g/1	

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In order to prepare the medium, the C source was added to one-times concentrated solution B and the mixture was sterilized by autoclaving. After the medium had cooled down, 1/100 of the volume of separately autoclaved solution A was added.

Example 2 Isolation of the GLY1 gene from Ashbya gossypii

In order to isolate the gene for threonine 25 the glycine-auxotrophic Saccharomyces aldolase, cerevisiae mutant YM 13F (SHM1:: HIS3 shm2:: LEU2 was transformed, after selection for gly1:: URA3) fluoroorotic acid, with an Ashbya resistance to gossypii gene library. The gene library consisted of 30 genomic DNA which had been partially digested with Sau3A and from which fragments of 8 - 16 kb in size had been isolated by density gradient centrifugation and ligated into the BamHI-cut vector Yep352. The transformants were first of all selected for uracil prototrophy. Selection for glycine prototrophy was carried out in a second step after replica plating. 25 glycineprototrophic clones were isolated from about 70,000 uracil-prototrophic clones. Curing of the transformants with the isolated plasmids retransformation demonstrated that the complementation was plasmidencoded. Whereas there was no measurable threonine aldolase activity (< 0.1 mU/mg of protein) glycine-auxotrophic Saccharomyces strain, it was possible to measure significant enzyme activity (25 mU/mg of protein) in the strains which were transformed with the isolated gene library plasmids. A sub-cloned 3.7 kb Hind III fragment which exhibited complementation was sequenced (Figure 2). A threonine aldolase-encoding gene which was homologous to Saccharomyces cerevisiae GLY1 was found.

15 Example 3 Overexpressing the GLY1 gene in Ashbya gossypii

In order to overexpress the GLY1 gene, it was vector pAG203 (cf. expression into the WO9200379). In this plasmid, the gene is under the 20 control of the TEF promoter and the TEF terminator (Figure 3). A gene for resistance to G418 functions as a selective marker in Ashbya gossypii. After Ashbya gossypii had been transformed with this plasmid and single-spore clones had subsequently been isolated, 25 because the spores are mononuclear and haploid, the threonine aldolase activity in the crude extract was then measured. Both when growing on glucose and when growing on soybean oil, at least ten-fold overexpression was measured in A.g.p.AG203GLY1 as compared with a 30 strain which had been transformed with the empty plasmid pAG203 (Figure 4).

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Example 4
Increasing riboflavin formation by overexpressing GLY1
and feeding threonine

Threonine was added to the medium in order to 5 check whether the threonine which is formed in the cell limits the formation of glycine by the overexpressed threonine aldolase. When 6 grams of threonine were added per liter when A.g.pAG203GLY1 was growing on glucose as the carbon source, the strain 10 approximately twice as much riboflavin as it did when 6 grams of glycine were added per liter (Figure 5). This effect was not observed when a wild type and a control strain which was transformed with the empty plasmid were tested. Analysis of the amino acids in the medium showed that only about 6 mM of the fed-in 52 mM threonine remained in the case of the GLY1 overexpresser and that, surprisingly, the concentration of glycine had increased from 2 mM to 42 mM. results demonstrated that glycine formation was limited 20 by threonine, that the overexpressed threonine aldolase was capable of functioning, that glycine which was formed intracellularly was more effective than glycine which was fed extracellularly, and that the fungal cells exported glycine massively. 25

Example 5
Inhibiting glycine export

30 If the threonine aldolase-overexpressing strain A.g.pAG203GLY1 was cultured on soybean oil instead of glucose, as in Example 4, the increase in riboflavin formation obtained when feeding threonine did not exceed that when feeding glycine (Fig. 6). However, analysis of the medium showed that the threonine had been degraded down to about 13 mM. There cannot, therefore, have been any limitation in the threonine. At the same time, it was found that the extracellular glycine

had increased from 2 to about 44 mM. All the glycine which had been formed by the fungus had therefore been exported into the medium. It was possible to inhibit this export by introducing glycine into the medium, a measure which then resulted in the riboflavin formation being substantially increased in association with the same uptake of threonine (Table 2). In order to rule out the possibility that it was only the glycine which had been introduced which was responsible for the increased production, as much glycine was introduced, in a control, as was ultimately formed, as glycine, in the experiment using glycine and threonine. This finding underlines the fact that glycine which is formed intracellularly is much more effective than glycine which is added extracellularly.

Example 6

Increasing the formation of riboflavin by selecting β -hydroxynorvaline-resistant mutants

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Since it was not the conversion of threonine into glycine but the synthesis of threonine which first of all limited glycine formation, the threonine analog β -hydroxynorvaline was used to search for resistant mutants. Radial growth was significantly inhibited on agar plates filled with minimum medium containing β -hydroxynorvaline. Mutants which grew more vigorously formed spontaneously at the edges of the colonies. Stable mutants which grew significantly more vigorously on the β -hydroxynorvaline minimal medium than did the parental strains (Fig. 8) were produced by isolating spores and selecting once again. Investigation of riboflavin formation indicated a marked increase in productivity. First, medium in minimal containing soybean oil, the strain HNV-TB-25 formed 41 \pm 11 mg of riboflavin/l whereas its parental strain only produced 18 \pm 3 mg/l. The progeny strain HNV-TB-29 also exhibits a marked increase, with a formation of 116 \pm 4 mg/l, as

compared with its strain of origin, i.e. Ita-GS-01, which only formed 62 \pm 10 mg/l.

Table 1: ¹³C-enrichment in the C atoms of serine, threonine and glycine following growth of A. gossypii ATC10895 on the given media and subsequent total hydrolysis of the resulting biomass (MM: minimal medium; YE: yeast extract; YNB: yeast nitrogen base; n.d.: not determined)

Medium		MM + 0.2 g of YE/l + 1 g of ethanol/l + 2.7 mg of "C2-serine/l	MM + 0.2 g of YNB/1 + 1 g of ethanol/1 + 2.6-mg of $^{13}C_1$ -serine/1
Serine	C ₁	1.1	4.9
	C_2	5.9 .	1.1
	C3	1.1	1.1
Threonine	C ₁	n.d.	39.0
	C ₂		1.1
,	C,		1.1
	C ₄		1.1
Glycine	C,	1.1	7.1
	C ₂	4.3	1.1

Table 2: Effect of supplementation with threonine and glycine on riboflavin formation when GLY1 is simultaneously being overexpressed

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Strain	Carbon source	t = 0 Supple- ment	<pre>t = 72 h Riboflavin [mg/l]</pre>	t = 72 h Gly [mM]	t = 72 h Thr . [mM]
	Soybean oil	80 mM Gly 50 mM Thr	22 ± 1	80 ± 2	42 ± 0
WT		130 mM Gly	18 ± 3	129 ± 2	n.d.
	Glucose	80 mM Gly 50 mM Thr	5 ± 1	80 ± 0	35 ± 0
		130 mM Gly	7 ± 1	126 ± 2	n.d.
	Soybean oil	80 mM Gly 50 mM Thr	31 ± 0	117 ± 2	11 ± 1
Ag pAG		130 mM Gly	20 ± 3	129 ± 1	n.d.
203 GLY1	Glucose	80 mM Gly 50 mM Thr	40 ± 1	113 ± 2	12 ± 0.7
		130 mM Gly	9 ± 1	129 ± 3	n.d.
			n d	- not de	tectable

n.d. = not detectable

Comments on the figures

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- Figure 1: Formation of riboflavin by the Ashbya gossypii strains ATCC 10895 (wild type, WT) and the AMPS-resistant mutant AMPS-MN-01 in the presence or absence of 6 g of glycine/l following growth on complete medium containing 10 g of soybean oil/l as the carbon source. The measured values were obtained from three independent experiments.
- Figure 2a: Gly 1 locus in the Ashbya gossypii genome.

 The clones GB 7-1 and GB 26-9, and also the
 3.7 kb Hind III subclone GB-26-9-6, comple
 ment the S. cerevisiae mutant. GB-26-9-6

 was sequenced entirely while GB 7-1 was

 sequenced in order to complete the C

 terminus of GLY1.
- 20 Figure 2b: Nucleotide sequence, and deduced amino acid sequence, of the A. gossypii GLY1 gene together with the flanking nucleotide sequence.
- 25 **Figure 3:** Diagrammatic depiction of the construction of the vector pAG203GLY1 for overexpressing the GLY1 gene in A. gossypii.
- Figure 4: Comparison of Ashbya gossypii wild type

 (solid symbols) and A.g.pAG203GLY1 (open symbols) with regard to growth, riboflavin formation and specific threonine aldolase activity when cultured on complete medium containing 10 g of soybean oil/1.
 - Figure 5: Growth and riboflavin formation of Ashbya gossypii strains ATCC 10895 (wild type), pAG203 and pAG203GLY1 when cultured on YE

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complete medium containing 10 g of glucose/l as the carbon source and in association with glycine or threonine supplementation. The Table shows the glycine and threonine concentrations in the medium in each case before and after culture. The mean values and standard deviations shown represent the results from three independent experiments.

- Growth and riboflavin formation of Ashbya 10 Figure 6: gossypii strains ATCC 10895 (wild type), and pAG203GLY1 when cultured on pAG203 complete medium containing 10 g of glucose/l as the C source and in association with glycine or threonine supplementation. The 15 Table shows the glycine and threonine conin the medium in each case centrations before and after culture. The mean values and standard deviations shown represent the results from three independent experiments. 20
- Comparison of Ashbya gossypii wild type Figure 7: (solid symbols) and the AMPS-resistant mutant AMPS-NM-01 with regard to growth, riboflavin formation and the specific 25 activities of threonine aldolase, hydroxymethyltransferase and glutamate glyoxylate aminotransferase when cultured on complete medium containing 10 g of soybean The measured values were obtained 30 from three independent experiments.
- Figure 8: Effect of β -hydroxynorvaline on Ashbya gossypii; growth of wild type (W) and HNV-TB-25 (H) on an agar plate which is filled with minimal medium containing 2.5 g of glucose/l and 2.5 mM β -hydroxynorvaline.

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- (ii) TITLE OF INVENTION: UNICELLULAR OR MULTICELLULAR ORGANISMS
 FOR PREPARING RIBOFLAVIN
- (iii) NUMBER OF SEQUENCES: 2
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 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Disk 3.5" / 1.44 MB
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: TXT ASCII
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: 2,255,284
 - (B) FILING DATE: December 17, 1998

(vii)	PRIOR	APPLICATION	V DATA:					
	(A)	APPLICATION	NUMBER:	DE	197	57	180.	8

- (B) FILING DATE: December 22, 1997
- (A) APPLICATION NUMBER: DE 198 40 709.2
- (B) FILING DATE: September 09, 1998
- (2) INFORMATION FOR SEQ ID NO: 1:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2744 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (iv) ANTI-SENSE: NO
 - (v) FRAGMENT TYPE: N-terminal
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Ashbya gossypii
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION:1232..2377
 - (C) IDENTIFICATION METHOD: experimental
 - (D) OTHER INFORMATION:/codon start= 1232 /product= "Threonin-Aldolase" /evidence= EXPERIMENTAL /number= 1
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTGCCATTAA	TGACCGGGAG	CCTGAAGGTG	TGTGATGAAC	AAGCCAGTCT	TCCCCGCGCG	60
TCGCCAACTG	CTCGTCATAT	AATCCCGGAA	AAGCTCGCAT	TAGGTGAAAT	TTTTCTTAGG	120
AATTACATCT	GCTACTGACA	AAACTAAGTA	AAAGCTCCGA	TAGGTAGCCG	TGCTGCCGAG	180
CACCTGCCTA	ATACACGCAG	GCGCCATACA	CTATTTAAGC	ACAATGTTAT	CGCCCGCAG	240
CTTGAGGTAT	TCCTGGTCGA	TGCCAGGTGT	CATAGGCTTG	ATCACCAGCG	AGTAGACCTC	300

ACTATTGTAG AAGCGCAGCC CGTTGCTGGG GGACTTGTAG CGCGCCTTGA GCCCCGTGAT	360
GTCGCAGTAG CGTTTCACGG GATACTGCGA TGGTGGCGCC TGAATGTTGA AGTATGTCAG	420
CTTCGTGCGC CCTGCGTCAC GCCCGGCTTC CGACTGTGCC TCTGTCGTGA GCCGTTTCCA	480
CTCGTCTGTC AGAAGCTGAC GTGTCGGCTT GTGGCGGCGC GTGGGTTTCT TCCACGTGGG	540
CGACTTGAAG TCGCTACGAC TGGTATCATT ACGTGCTGCA ATCGCTCGGA GGTTCTCCAT	600
CTGGGGTCCA CGGTCGCTCG TTGATCTGTC TATCTCGAAA TCCCTGCCCA GATGTACTCC	660
CATGTTATCA CGTGACCACA CGCCGTTTTC GTGTGTAGTG ATGCAGATGG TTCTAGAGCA	720
TCACGTGGCT TACATAGCTT TGTTACATAA TCGATTTTCC GCAGGAGCGT TACGTCCAAC	780
GGTCGTTCTG TGCCAAAAGC AACAACTGAG CGTCAGGCGG CCGTCTCCCC AGACACGCTC	840
CGCCCCAAAC TGAGCTCCAC GCGGCCTTCT GTCCGAGTTA AGTTCCTCCC CGCTCGTCAG	900
CACGGGGTCT TTCGTCGCCT ATCCTCCTGC AGCGTTCGCT ACTGCAGATC GTGAGCAGTG	960
GCACCCGCGA CCAAAAAAAG AAATTATGTT CCTTACGCAA GGAATATGCC TCGCGCCATG	1020
CCATCGCAAA GAGTGATGCC GCAGAGGTTG CTTCTGCGAG GCAACTCCTG GGCAATAGGG	1080
TGGAAAATTC AGCTTGGGCT TATATAAAAG AAACCGTTCG AGCTCGTCGG AGCCAGGTGG	1140
AAAATTTTTC GTAACGTAGG TAGAGGTTAT AGTTAGCGTC AGTCTCTTTT CTGCCAAGCT	1200
GCTACAGTTG ACTACAAGTA ACAAACCCAG G ATG AAT CAG GAT ATG GAA CTA Met Asn Gln Asp Met Glu Leu 1 5	1252
CCA GAG GCG TAC ACG TCG GCT TCG AAC GAC TTC CGT TCG GAC ACG TTC	1300
Pro Glu Ala Tyr Thr Ser Ala Ser Asn Asp Phe Arg Ser Asp Thr Phe 10 15 20	
ACC ACT CCA ACG CGC GAA ATG ATC GAG GCT GCG CTA ACG GCG ACC ATC Thr Thr Pro Thr Arg Glu Met Ile Glu Ala Ala Leu Thr Ala Thr Ile	1348
25 30 35	•
GGT GAC GCC GTC TAC CAA GAG GAC ATC GAC ACG TTG AAG CTA GAA CAG Gly Asp Ala Val Tyr Gln Glu Asp Ile Asp Thr Leu Lys Leu Glu Gln	1396

40			45			50			55	
			GCC Ala							1444
			CAG Gln							1492
			TGC Cys							1540
			ATT Ile							1588
			TAC Tyr 125							1636
			GAC Asp							1684
			CAC His							1732
			TGT Cys							1780
			AAC Asn							1828
			TTC Phe 205							1876
			GGC Gly							1924

Page 4

					TTC Phe											1972
					AAG Lys										_	2020
					CGT Arg											2068
					GGC Gly 285											2116
					TTG Leu											2164
					AAG Lys											2212
					ATA Ile											2260
					TTC Phe											2308
					ATC Ile 365											2356
					AAG Lys		TAA	GGA.	TTT (CGATO	GATG	AC A'	rgaa <i>i</i>	\AAT'	Г	2407
ACA'	TATT	GGC A	ACGG(CATAC	GG CI	ATTGO	GTA	A TA	PAATT	GCAT	ATG	GTTG	AGA '	rgaa'	r ta ctg	2467
TTC	GGT	ACC (GTA:	TTTC	CA A	AGTG	CTGT	C GA	CTTT:	rgca	AGA	GATG	GCT A	ATGA	ATGGGG	2527

Page 5

CACGCTCCAT	CACCTCTCTG	CGAGCCGGAC	TCAGCATTAT	ATCCATCTCA	AAACCTAATA	2587
TCAAATGGGA	TTGTGGTGCG	CAGTACATGC	GCAGTGCTGC	ACATTTGAGG	ATCAATGGGT	2647
TTTTCCAGGC	ACTGCCTGGG	TCACTCACCC	TATTGCGGAG	GGACTAGTAG	CTCTACCATT	2707
CTGAGCTGAC	TAAAATGTTT	GATTCTTTTG	GTACTTA			2744

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 382 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Asn Gln Asp Met Glu Leu Pro Glu Ala Tyr Thr Ser Ala Ser Asn 1 5 10 15

Asp Phe Arg Ser Asp Thr Phe Thr Thr Pro Thr Arg Glu Met Ile Glu 20 25 30

Ala Ala Leu Thr Ala Thr Ile Gly Asp Ala Val Tyr Gln Glu Asp Ile 35 40 45

Asp Thr Leu Lys Leu Glu Gln His Val Ala Lys Leu Ala Gly Met Glu 50 55 60

Ala Gly Met Phe Cys Val Ser Gly Thr Leu Ser Asn Gln Ile Ala Leu 65 70 75 80

Arg Thr His Leu Thr Gln Pro Pro Tyr Ser Ile Leu Cys Asp Tyr Arg 85 90 95

Ala His Val Tyr Thr His Glu Ala Ala Gly Leu Ala Ile Leu Ser Gln
100 105 110

Ala Met Val Thr Pro Val Ile Pro Ser Asn Gly Asn Tyr Leu Thr Leu 115 120 125

Glu Asp Ile Lys Lys His Tyr Ile Pro Asp Asp Gly Asp Ile His Gly

130 135 140

Ala Pro Thr Lys Val Ile Ser Leu Glu Asn Thr Leu His Gly Ile Ile His Pro Leu Glu Glu Leu Val Arg Ile Lys Ala Trp Cys Met Glu Asn Asp Leu Arg Leu His Cys Asp Gly Ala Arg Ile Trp Asn Ala Ser Ala Glu Ser Gly Val Pro Leu Lys Gln Tyr Gly Glu Leu Phe Asp Ser Ile Ser Ile Cys Leu Ser Lys Ser Met Gly Ala Pro Met Gly Ser Ile Leu Val Gly Ser His Lys Phe Ile Lys Lys Ala Asn His Phe Arg Lys Gln Gln Gly Gly Gly Val Arg Gln Ser Gly Met Met Cys Lys Met Ala Met Val Ala Ile Gln Gly Asp Trp Lys Gly Lys Met Arg Arg Ser His Arg Met Ala His Glu Leu Ala Arg Phe Cys Ala Glu His Gly Ile Pro Leu Glu Ser Pro Ala Asp Thr Asn Phe Val Phe Leu Asp Leu Gln Lys Ser Lys Met Asn Pro Asp Val Leu Val Lys Lys Ser Leu Lys Tyr Gly Cys Lys Leu Met Gly Gly Arg Val Ser Phe His Tyr Gln Ile Ser Glu Glu Ser Leu Glu Lys Ile Lys Gln Ala Ile Leu Glu Ala Phe Glu Tyr Ser Lys Lys Asn Pro Tyr Asp Glu Asn Gly Pro Thr Lys Ile Tyr Arg Ser Glu Ser Ala Asp Ala Val Gly Glu Ile Lys Thr Tyr Lys Tyr

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370 375

Claims

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- 1. A unicellular or multicellular organism, in particular a microorganism, for the biotechnological preparation of riboflavin, which exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 q of external glycine/1.
- 2. A unicellular or multicellular organism as claimed in claim 1, in which the intracellular synthesis of glycine is increased and/or the intracellular degradation of glycine and/or the transport of glycine out of the cell is at least partially inhibited.
- 3. A unicellular or multicellular organism as claimed in claim 1 or 2, which exhibits an increased threonine aldolase activity.
- 20 4. A unicellular or multicellular organism as claimed in one of claims 1 to 3, in which the intracellular formation of serine from glycine is at least partially blocked.
- A unicellular or multicellular organism as
 claimed in claim 4, in which the activity of serine hydroxymethyltransferase is at least partially blocked.
 - 6. A unicellular or multicellular organism as claimed in claim 4 or 5, which is resistant to glycine antimetabolites.
- 7. A unicellular or multicellular organism as claimed in claim 6, which is resistant to alpha-aminomethylphosphonic acid or alpha-aminosulfonic acid, β -hydroxynorvaline and/or other threonine and/or lysine analogs.
- 35 8. A unicellular or multicellular organism as claimed in any one of claims 1 to 7, which is a fungus, preferably from the genus Ashbya.

- 9. A unicellular or multicellular organism as claimed in one of claims 1 to 8, which is a fungus of the species Ashbya gossypii.
- 10. A threonine aldolase gene having a nucleotide sequence which encodes the amino acid sequence shown in Figure 2b and its allelic variantion.
 - 11. A threonine aldolase gene as claimed in claim 10 which has the nucleotide sequence of nucleotide 1 to 1149 as depicted in Fig. 2b or a DNA sequence which has essentially the same effect.

10

- 12. A threonine aldolase gene as claimed in claim 10 or 11 which possesses an upstream promoter having the nucleotide sequence from nucleotide -1231 to -1 as depicted in Fig. 2b or a DNA sequence which has essentially the same effect.
- 13. A threonine aldolase gene as claimed in one of claims 10 to 12 together with regulatory gene sequences which are assigned to this gene.
- 14. A gene structure which contains a threonine 20 aldolase gene as claimed in one of claims 10 to 13.
 - 15. A vector which contains a threonine aldolase gene as claimed in one of claims 10 to 13 or a gene structure as claimed in claim 14.
- 16. A transformed organism for preparing riboflavin 25 which contains, in replicatable form, a threonine aldolase gene as claimed in one of claims 10 to 13 or a gene structure as claimed in claim 14.
 - 17. A transformed organism as claimed in claim 16 which contains a vector as claimed in claim 15.
- 30 18. A process for preparing riboflavin, which comprises employing an organism as claimed in one of claims 1 to 9.
- 19. A process for preparing a riboflavin-producing unicellular or multicellular organism which comprises altering the organism such that it exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the

species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/1.

20. The process as claimed in claim 19, wherein the organism is altered using genetic engineering methods.

- 21. The process as claimed in claim 19 or 20, wherein the alteration of the organism is achieved by exchanging the promoter and/or increasing the gene copy number.
- 10 22. The process as claimed in one of claims 19 to 21, wherein an enzyme possessing increased activity is produced by altering the endogenous threonine aldolase gene.
 - 23. The process as claimed in one of claims 19 to
- 15 22, wherein the activity of the serine hydroxymethyltransferase is at least partially blocked by altering the endogenous serine hydroxymethyltransferase gene.
 - 24. The use of the organism as claimed in one of claims 1 to 9 and 16 and 17 for preparing riboflavin.
- 20 25. The use of the threonine aldolase gene as claimed in one of claims 10 to 13 and the gene structure as claimed in claim 14 for preparing an organism as claimed in one of claims 1 to 9 and 16 and 17.
- 25 26. The use of the vector as claimed in claim 15 for preparing an organism as claimed in one of claims 1 to 9 and 16 and 17.

Drawings

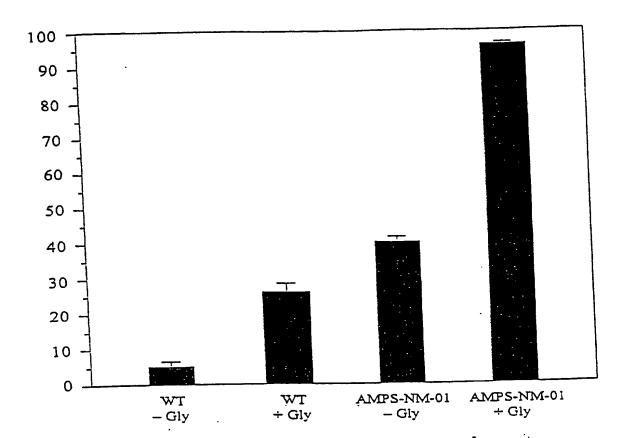


Fig. 1

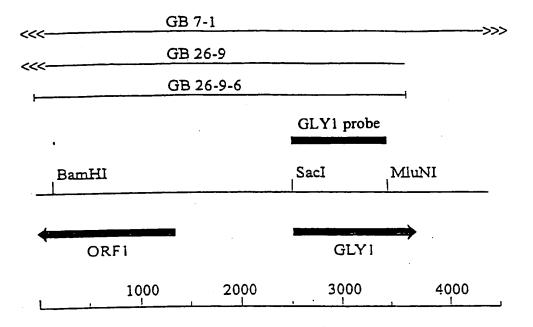


Fig. 2a

1/2

Sequences

								T TO	CCAT	TAAT	GAC	CGGG	AGC	CTG	AAGG:	IGI -	1201
GTO	ATGA	ACA	AGCC	AGTO	TT C	ccce	CGCC	כר כנ	SCCAR	CTGC	: TCG	ITCAI	'ATA	ATC	GG	<u>, , , , , , , , , , , , , , , , , , , </u>	-1141
AGG	TCGC	TTA	AGG1	GAA	TT I	TTCI	TAGO	3A A1	ITACA	ATCTO	CTA	VCTGA	CAA	AAC:	raag:	raa .	-1051
320	SCTCC	CAT	AGG	'ልፍርር	GT G	CTGC	CGAC	SC AC	CTGC	CTA	TAC	:ACGC	AGG	CGC	CATA	CAS .	-1021
TAT	TTA	LGCA	CAAS	GTT	FIC C	SCCCC	CGCAC	GC T	IGAGO	STAT	CCI	IGGTC	GAT	GCC	AGGT	31C	-961
ATA	GGC1	ADT.	TCAC	CAG	GA C	JTAG/	ACCT	EA C	TATTO	JTAG	AGC	COCAC	,000	GII	5013	-5 T	-901 -341
GA	CTTGT	CAGC	GCGG	CTT	SAG (STGA:	IG TO	CGCA	STAG	CT		955		C	eri Tee	-781
GG'	TGGC	SCCT	GAA	CCT	38 <u>4</u> (TAIL		שר ז מר א	CCTC	ひいいいい	2 C10	CCTC	200	TOT	CGC.	TTG	-721
GA.	CTGT	SCCT	CTG	regre	ans (STOCK	BC B	アレエスに	233C.	r cea		200	GGT	בים דים: בים דים:	TTE	-661
TG	TGCT(-656	TOG	31111 TTCC	53G (TCCA	TO TO	GGGG	TCCA	c GG	TCGC:	CGT	TGA	TCTG	TCT	-601
7.0	CTCG	7 A A E	103	TECC	CAG	ATGT!	LCTC	כר ג	TGTT.	ATCA	CGT	GACCI	ACAC	GCC	GTTT	TCG	-541
TC	TCTA	CTCA	TGC	ひたたか	GGT '	TOTAL	GAGC	AT C	ACGT	GGCT	TAC.	ATAG	TTT	GTT	PS_AT	PA1	-481
	B TTT	TOCG	C2.C	GAGC	GTT :	PCGT(CCAA	cg e	TCGT	TCTG	TGC	CAPA	ag:ta	ACA	ACTG	_انايخر	-421
GT	CAGG	cesc	CGT	CTCC	CCA	GACA	CGCT	CC G	cccc	AAAC	T GA	GCTC:	CACG	CGG	CCTT	CIG	-361
-	~~~~	TTER	CTT	-crc	ccc	CCTC	スカナシ	CC A	casa	GTCT	TTC	GTCG	CCTA	TCC	TECT	GCA	-301
cc	CTTC	CCTL	CTG	CAGA	TCG	TGAG	CAGT	GG C	accc	GCGA	C CA	AAAA.	aaga.	AAT	TATG	TTC	-241
СТ	TACG	CAAG	GAA	TATG	CCT	CGCG	CCAT	GC C	ATCG		G AG	TGAT	فاحات	CAG	いいししょ	-101	-161
TT	CTGC	gagg	CAA	STCC	TGG	GCAA	TAGG	GT G	GALA	ATTC	A GC	TTGG	GCTT	ATA	AAAT	PLEA.	-121
وع	CCGI	TCGA	. GCT	CGTC	GGA	GCCA	GGTG	GA A	TTAA	TTTC	G TA	ACGT.	AGG T	المحالية	6611	AIA	-61 -1
GT	TAGC	GTCA	GTC	TCTT	TIC	TGCC	AAGC	TG C	TACA	GTTG	A CT	ACAA	ولينز لاجا	ريني .	MUCC	.AGG	-1
								cc3	c	ccc	TEC	ACC.	TCG	CCT	TCG	220	48
	ATG Met	AAT	CAG	Gril	ATG	بدين	CIA	CCA.	Clas	21-	The	75 -	Ser	Al=	Ser	Asn	• •
1	Met	Asn	GIR	ASP	Mec	Gili	Leu	PIO	Giu	ALA	17.		341				
	GAC		CCT	TCG	G2C	ACG.	TT	ACC.	200	CCA	ACG	CGC	GA B	ATG	ATC	GAG	96
	ASP	710	7	507	Aen	75-	Phe	アカテ	The F	D-n	The	Arg	Glu	Met	Ile	Glu	
17	ASP	Pne	وعم	26-	بردم	****	Z					,					
	~~~	GCG	CTA	¥CG	GCG	ACC	ATC	GGT	GAC	GCC	GTC	TAC	CAA	GAG	GAC	ATC	144
33	Ala	Ala	Leu	Thr	Ala	Thr	Ile	Glv	ASP	Ala	Val	Tyr	Gln	Glu	<b>QZA</b>	Ile	
	GAC	ACG	TIG	AAG	CTA	GAA	CAG	CAC	GTC	GCC	AA.G	CTG	GCC	GG€	ATG	GAG	192
49	Asp	Thr	Leu	Lys	Leu	Glu	Gln	Ris	Val	Ala	Lys	Leu	Ala	Glā	Met	Glu	
	_																240
	GCC	GGT	ATG	TTC	TGC	GTA	TCT	GGT	ACT	TTG	ICC	AAC	CAG	ATT	GCT	TTG	240
€5	Ala	CJA	Met	Phe	Cys	Val	Ser	Gly	Thr	Leu	Ser	Asn	GIN	ITe	YT#	Leu	
			·								»	CTT	TCC	CAC	TAC	CGT	288
	CGG	ACC	CAC	CTA	ACT Thr	CAG	CCA	CCA	T.A.I	For	Tin	Tan	CVE	Asn	TVT	Ara	
81	Arg	Thr	CIÃ	Leu	TEF	Gin	PIO	PIO	TAL	Ser	110	Leu	Cys	وصر	-1-	,	
		C 7 T	-	TEC	ACG	CAC	GAG	CCT	cce	ccc	TTG	GCA	ATT	TTG	TCC	CAG	336
	31-	CA.	37-1	The	Thr	Fie	Gin	Ala	Ala	Glv	Leu	Ala	Ile	Leu	Ser	Gln	
97																	
	GCC	ATG	GTG	ACA	CCT	GTC	ATT	CCT	TCC	AAC	GGC	AAC	TAC	TTG	ACT	TIG	384
113	Ala	Met	Val	Thr	Pro	Val	Ile	Pro	Ser	Asn	Gly	Asn	Tyr	Leu	The	Leu	
-1-																	
	GAA	GAC	ATC	AAG	AAG	CAC	TAC	ATT	CCT	GAT	GAT	GGC	GAC	ATO	CAC	GGT	432
129	Glu	Asp	Ile	Lys	Lys	His	Tyr	Ile	Pro	Asp	Asp	Gly	Asp	Ile	His	Glā	•
	GCT	CCA	. ACA	. AAG	GTT	ATC	TCG	TTG	GA.A	AAC	: ACC	TTG	CAC	GG:	ATO	ATI	-
145.	ALa	Pro	Thr	Lys	Val	Ile	Ser	Leu	ı Glu	מבאו	Thr	Leu	Eis	CT?	, ITE	TTE	}
	CAC	CCA	CTA	GAG	GAG	CIT	GII	. cea	ATC	: AAG	GCT	TGG	TGI	ALL	- C1.		
161	His	Pro	Leu	Glu	Glu	Leu	Val	. Arg	Ile	Lys	. Ala	Trp	Cys	Me	الما ع	, ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	•
			<b>_</b>								3.70			- 60	TO	GCE	576
	GAC	CTC	AGA	CTA	CAC	TGL	. GA:	GGI		, Alex	. 710	. 100	No.	A1:	501	- Ala	
177	Asp	Leu	Arg	Let	Eis	cys	A5)		, wrs	LATO	, 116	, trb	- ASI	. ~			-
	<b></b>	TC-			CCI		232		- TAC		620	CT2	TTO	GA/	I TO	AT:	624
100	ج _ي دي Glu		. 607 - 10	. V-1	D	. Car	i lar	. <u></u>	n Tur	. Gl:	, Glu	Leu	Phe	A.5	Se:	- 110	a
193																	
	TOO	P.TC	TG	770	TCC	AAC	TCC	ATO	GG1	GCC	CCF	ATG	GGG	TC	TA D	r cr	672
209	Ser	Ile	CV	Lei	Se:	Lvs	Se:	Me	E Gly	Ala	Pro	Met	G13	, Se	r Ile	e Le	د
			,-			-2-											

#### 2/2

225	GTC Val	<b>C</b> TA GGC	TCG Ser	CAC His	AAG Lys	TTC Phe	ATA Ile	AAG Lys	AAG Lys	GCG Ala	AAC neA	CAC His	TTC Phe	AGA D=4	AAG Lys	CAG Gln	720
241		GLY GLY	C1A C21	eja eei	GTC Val	AGA Arg	CAG Gln	TCT Ser	GGT Gly	ATG Met	ATG Met	TGC Cys	AAG Lys	ATG Met	GCG Ala	ATG Met	768
257		GCT Ala	ATC	CAG Gln	Gly Gly	GAC <b>Asp</b>	TGG Trp	AAG Lys	GGC	AAG Lys	ATG Met	agg Azg	CGT	TCG Ser	CAC His	AGA Arg	816
273	ATG Met	GCT Ala	CAC His	GAG G1 u	CTG.	GCC Ala	AGA Arg	TTT Phe	TGC Cys	GCA Ala	GAG Glu	CAC His	GGC Gly	ATC Ile	CCA Pro	TTG Leu	964
289	GAG Glu	TCG Ser	CCT Pro	GCT Ala	GAC <b>A</b> sp	ACC Thr	AAC Asn	TTT Phe	GTC Val	TTT Phe	TTG Leu	GAC Asp	TTG Leu	CAG Gln	AAG Lys	AGC Ser	912
305	A.A.G Lys	ATG Met	AAC Asn	CCT Pro	GAC Asp	GTG Val	CTC Leu	GTC Val	AAG Lys	AAG Lys	AGT Ser	TTG Leu	AAG Lys	TAC Tyr	ejā eec	TGC Cys	960
321	AAG Lys	CTA Leu	ATG Met	GT A	<b>e</b> ta eee	CGT Arg	GTC Val	TCC Ser	TTC Phe	CAC His	TAC	CAG Gln	ATA Ile	TCT Ser	GAG Glu	GAG Glu	1008
337		CTT Leu	GAG <b>G</b> lu	AAG Lys	ATC Ile	AAG Lys	CAG Gln	GCC	ATC	CTA Leu	GAG	GCG	TTC Phe	GAG <b>Gl</b> u	TAC Tyr	TCG Ser	1056
353	AAG Lys	AAG Lys	AAC Asn	CCT	TAC	GAT <b>As</b> p	GAA Glu	AAC neA	GGC	CCC	ACG	AAG Lys	ATC Ile	TAC Tyr	AGA Arg	AGT Ser	1104
369	GAG Glu	TCC	GCI	GAC Asp	GCT	GTG Val	GGT	GAG	ATC	AAG Lys	ACC Thr	TAC	AAG Lys	TAI	TAA	•	1149
	GGGAT TAAGC TTTTG GCATI GTGCT	TTCC ATAI CAAC ATAI	IA TO IG GT IA GA IC CA	ATGA TGAC TGGC ATCTO	CATO ATGA TATO AAAA	AAA ATT AAT CCT	AATT ACTO GGGO AATA GGGO	ACA TTC CAC TCA	TATT GGGT GCTC AATC	GGCP PACCO CATO GGA1	ACG G	CATA ATTTO CTCTO PGGTO SCCTO	AGGCA CAAA TGCG GCGCA	T TO	GGTA CTGT CGGA CATG	CTCA	1209 1269 1329 1369 1449 1509

Figure 2b

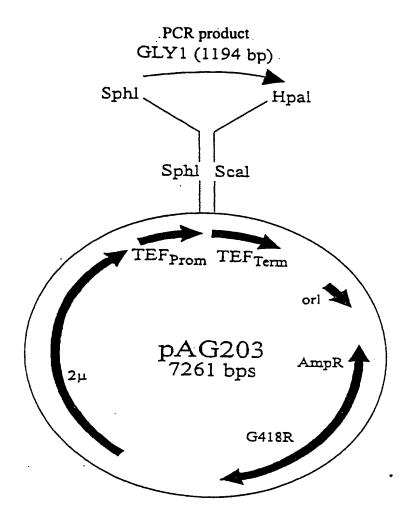


Fig. 3

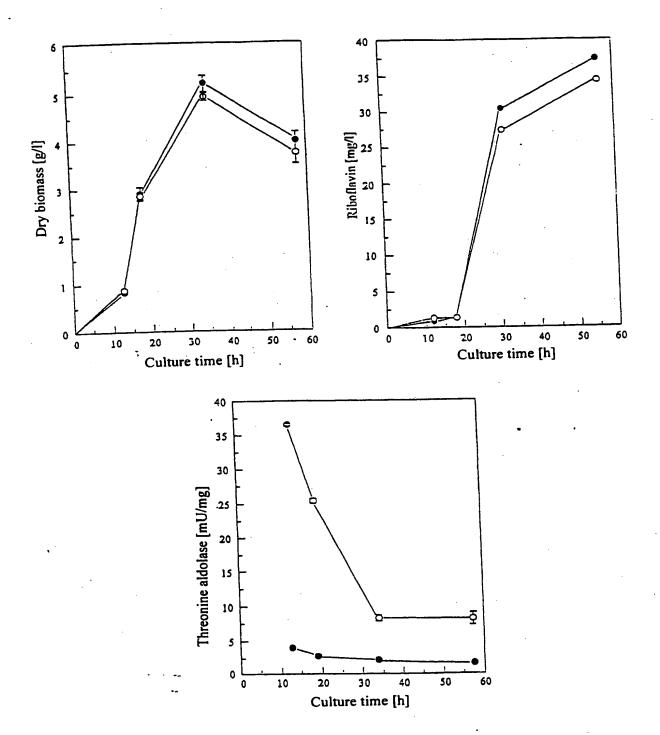
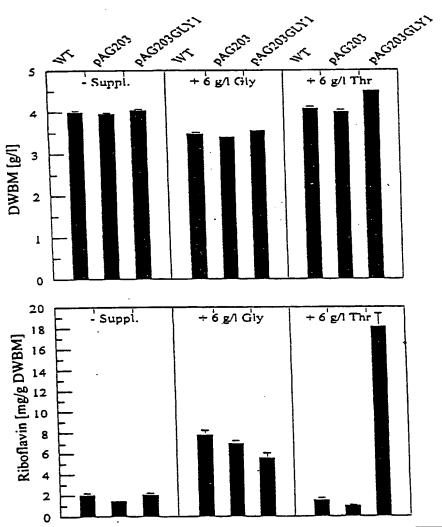
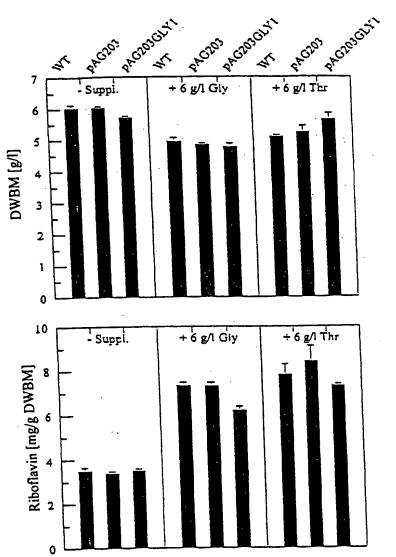


Fig. 4



Strain	Medium	Before	culture	After culture					
		Gly [mM]	Thr [mM]	Gly [mM]	Thr [mM]				
A. g. WT	-	2	1.6	$2.3 \pm 0.04$	0.18 ± 0.08				
	7+6 g of Gly/l	82	1.6	$79.6 \pm 0.8$	$1.2 \pm 0.1$				
	+6g of Thr/l	2	51.6	$6.3 \pm 0.3$	$32.0 \pm 1.2$				
A. g. pAG203GLY1	.	2	1.6	$4.0 \pm 0.08$	$0.14 \pm 0.01$				
<i>8</i> . <b>F</b>	+6g of Gly/l	82	1.6	$80.2 \pm 0.7$	$0 \pm 0$				
	+6 g of Thr/l	. 2	51.6	$41.3 \pm 0.7$	$6.1 \pm 1.0$				

Fig. 5



Strain	Medium	Before culture		After culture	
		Gly [mM]	Thr [mM]	Gly [mM]	Thr [mM]
A. g. ATCC 10895		2	1.6	$2.4 \pm 0.03$	0 ± 0
	+ 6 g of Gly/l	82	1.6	$76.5 \pm 0.4$	$0 \pm 0$
	+ 6 g of Thr/l	2	51.6	$5.6 \pm 0.7$	$42.8 \pm 1.0$
A. g. pAG203GLY1	-	2	1.6	$4.0 \pm 0.06$	$0 \pm 0$
	+6 g of Gly/l	82	1.6	$78.3 \pm 2.0$	$0 \pm 0$
	+.6 g of Thr/l	2	51.6	$44.0 \pm 4.1$	$12.6 \pm 1.8$

Fig. 6

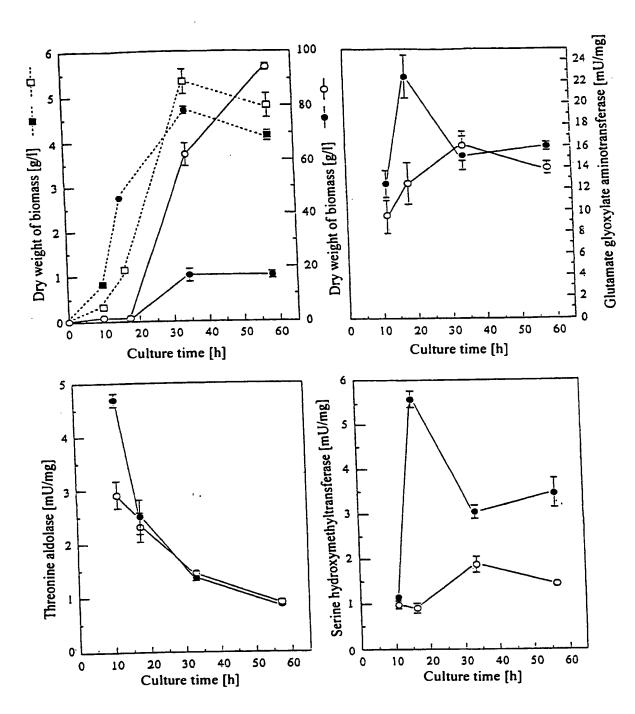


Fig. 7

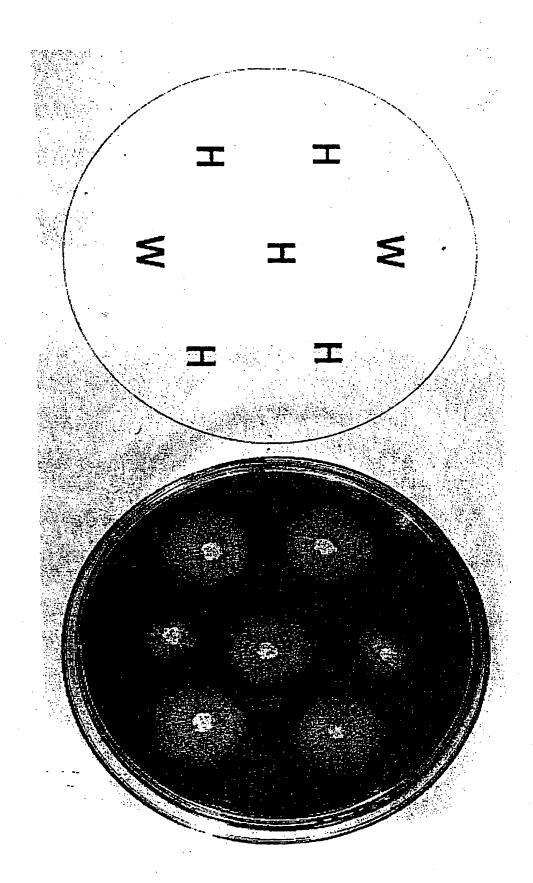
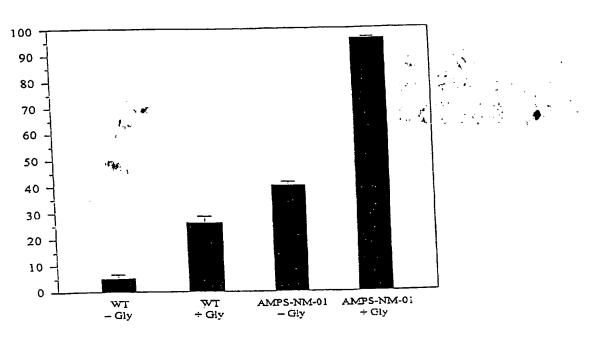


Fig. 8

#### Drawings



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